

Potential anti-cervical carcinoma drugs with agonist and antagonist AhR/PXR activities

Authors:

Wissem Mnif^{1,2,*}, Ines Zidi³, Patrick Balaguer⁴

¹ Univ. Manouba, ISBST, BVBGR-LR11ES31, Biotechpole Sidi Thabet, 2020, Ariana, Tunisia.

² Faculty of Sciences and Arts in Balgarn PO BOX 60 Balgarn- Sabt Al Alaya 61985, Bisha University, Saudi Arabia.

³ Laboratory Microorganismes et Biomolécules Actives, Sciences Faculty of Tunis, Tunis El Manar University, 2092 Tunis, Tunisia,

⁴ IRCM, Institut de Recherche en Cancerologie de Montpellier, CRLC Val d'Aurelle Paul Lamarque, INSERM, U896 34298 Montpellier, France; Université Montpellier 1, 34298 Montpellier, France.

* *Corresponding author:*

Dr. Wissem Mnif

LR11-ES31 Laboratory of Biotechnology and Valorisation of Bio-GeoRessources, Higher Institute of Biotechnology of Sidi Thabet, BiotechPole of SidiThabet, 2020, University of Manouba, Tunisia.

E-mail: w_mnif@yahoo.fr

Tel: + (216) 98 94 73 71

Abstract

Cervical cancer (CC) is responsible for higher morbidity and mortality in the world. To understand the pharmacologic drugs' efficacy in CC therapy, we investigated the ability of some widely used or potential anti-cancer drugs (alpha-naphthoflavone (ANF), beta-naphthoflavone (BNF), clotrimazole (CLO), dimethoxybenzoquinone (DMB), paclitaxel (PAC), rifampicin (RIF), and RU-486) to agonize or antagonize with nuclear receptors: aryl hydrocarbon receptors (AhR) and pregnane X receptors (PXR). To achieve this goal, we performed our study on positive transfected HeLa cell lines, a representative cell line model for CC. In this paper, a study of the recent patents for the importance of these drugs as AhR or PXR agonists or antagonists was evaluated. However, we demonstrated that ANF and PAC were found to agonize the AhR-mediated transcription in a dose-dependent manner. We reported also that PAC and RIF induced PXR, a strong agonist activity. Antagonistic activities toward AhR and PXR were shown only in BNF. Because little is known about the implication of AhR and PXR in cancer therapy, our results could be helpful for the design of future CC therapeutics. Future studies could lead to a better understanding of AhR and PXR transactivation/inhibition implication in CC; and lead to a better knowledge of CC therapy.

Key words: Cervical cancer; therapy; AhR; PXR; alpha-naphthoflavone; beta-naphthoflavone; clotrimazole, dimethoxybenzoquinone; paclitaxel; rifampicin; RU-486.

1. INTRODUCTION

Cervical cancer (CC) is the most devastating gynecologic malignancy in the world [1]. Its screening becomes compulsory to avoid invasion and deleterious effects. This malignancy is closely associated to viral infections especially human papillomavirus (HPV), as well as early age and frequent sexual contact [1,2]. The standard cancer therapies available are surgery, radiotherapy (usually teletherapy associated to brachytherapy), and chemotherapy. In addition, several prevention therapeutics are proposed for CC including therapeutic vaccines, chemo-preventive drugs, retinoids, indole carbinol, and immune modulators agents [1].

Radical hysterectomy (Meigs-Wertheim hysterectomy) stills the most traumatic therapy for women. For this reason, the use of novel therapeutics is preferred to favor good patients' outcome with limited surgery. For a long time, CC has been considered as not cured only by chemotherapy, however new clinical studies are proving the improvement of patients' responses with the use of

carboplatin, iphosphamide, 5-fluorouracile, doxorubicin, methotrexate, hexamethylmelamine, mitomycin-C, vinblastine, bleomycin, paclitaxel, topotecan, vinorelbine, and irinotecan [3]. The combination of drugs might be a solution to delay possible recurrence and change ideas about pharmacotherapy.

In this study, we compared the response of aryl hydrocarbon receptors (AhR) and pregnane X receptors (PXR) in HeLa human CC cell line to some used and potential anti-cancer drugs including alpha- (ANF) and beta-naphthoflavone (BNF), clotrimazole (CLO), dimethoxybenzoquinone (DMB), paclitaxel (PAC), rifampicin (RIF), and RU-486 (Figure 1). We have tested receptors transactivation level. We classified anti-cancer drugs as AhR or PXR agonists or antagonists (Table 1). Our study could be helpful for the design of new CC therapies. Further clinical studies should concentrate on the effect of these drugs combination during CC therapy.

2. MATERIALS AND METHODS

2.1. Materials

The cell culture medium and other additives were obtained from Life Technologies (Cergy-Pontoise, France). Luciferin was from Promega (Saint-Quentin-Fallavier, France). The RIF and CLO were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). RU-486 was gifts from Aventis (Romainville, France). The ANF, BNF, DMB, and PAC were purchased from Sigma Chemical Co (St. Louis, MO, USA). SR12813 was purchased from NEN Life Science Products (Paris, France). Dioxin was obtained from Promochem (Molsheim, France). For bioassays experiments, stock solutions were prepared in dimethyl sulfoxide (DMSO) at 10 mM and stored at -20°C.

2.2. Generation of stable transfected reporter cell lines and cell culture conditions

The stable transfected luciferase reporter cell lines were obtained as previously described [4].

The HahLP cell line was obtained by transfecting HeLa cells with the

CYP1A1-Luc and pSG5-puro plasmids. The selection of resistant clones was performed using 0.5 µg/ml puromycin. The strongest dioxin responsive clone was named HAhLP 1.15 (named here AhRE 1.15) [5].

The HGPXR reporter cell line was obtained by transfection of HeLa cells with the (Gal4RE) 5-βglob-Luc (HG5LN cells) and Gal4 (DBD)-hPXR (LBD) plasmids. Resistance of clones to puromycin and geneticin were performed at 0.5 µg/ml and 1 mg/ml, respectively. The most responsive clone was isolated and called HGPXR 4.3 (named here PXR4.3) [6].

The HahLP, and HGPXR cell lines were grown in phenol red containing Dulbecco's Modified Eagle's Medium (DMEM), 1 g/l glucose supplemented with 5% of fetal calf serum (FCS) and 1% antibiotics under a humidified 5% CO₂ atmosphere at 37°C. Taking estrogenic activities of FCS and phenol red into account, *in vitro* experiments were achieved in DMEM, without phenol red supplemented with 6% dextran-coated, charcoal-treated FCS (DCC) and 1% antibiotics (penicillin / streptomycin) (test

culture medium).

2.3. Luciferase assay

5×10^4 cells per well were seeded in 96-well plates in 150 μ l test culture medium. Tested extracts were 4x concentrated in the same medium. After 8 hr of cell seeding, 50 μ l were added. The cells were incubated with drugs during 16 hr. All experiments were performed in quadruplicates. At the end of the incubation, the effector-containing medium was removed and replaced by 3×10^{-4} M luciferin containing test culture medium. At this concentration, luciferin diffuses into the cell and produces a luminescent signal stable for 5 min and measured for 2 seconds by a Microbeta Wallac luminometer.

Drugs agonistic activities were expressed as the percentage of the maximal activity obtained with the reference ligand at saturating concentration: 10 nM for dioxin, and 1 μ M for SR12813, in, HahLP, and HGPXR cells, respectively. Tests were also performed to detect antagonistic activities of drugs. To achieve this goal, cells were activated with the reference agonist at a concentration yielding 80% of the

maximal activity in the presence of increasing concentrations of drugs.

3. RESULTS

To better define the affinity/activity of some anti-cancer drugs to AhR or PXR, we performed in vitro AhR and PXR assays on AhR-positive cell line: AhRE 1.15, and on PXR-positive cell line: PXR 4.3 (Table 1). Both cell sensitivity and reproducibility of the assay were assessed after the measure of AhR or PXR activators response (Figure 2). Measurements were taken for each well (triplicate wells for each dose) and the values represented the mean \pm S.D. of three independent experiments. Results of HAhLP and HGPXR bioassay were expressed in luciferase activity percentage, with a 100% transactivation value referring ligand at saturating concentration.

We demonstrated that ANF and PAC are high affinity agonists for AhR with 100% of transactivation at $<10^{-5}$ M. However, BNF is a moderate affinity agonist AhR with only 50% of effect at $>10^{-6}$ M, but in the same time a high affinity antagonist for AhR (100% of

effect at $<10^{-5}$ M). Finally, DMB showed low affinity for AhR with low effect at 10^{-5} M.

We reported also that PAC and RIF strongly activate PXR in PXR 4.3 cell lines. However, ANF, CLO and RU-486 are moderate PXR agonists. BNF and DMB are low affinity PXR agonists. We described in Table 1 also PXR antagonists. In fact, BNF are capable to strongly antagonize with PXR (100% of effect at $<10^{-5}$ M). However, ANF, as well as DMB exhibit low antagonist effect on PXR (low effect at 10^{-5} M).

4. DISCUSSION

Transactivation of AhR, a ligand-activated transcription factor of the helix-loop-helix/PAS family, is controversial in cancer context [7,8]. Indeed, some authors reported their implication in carcinogenesis, whereas evidence for the essential role of AhR as a key element for anti-cancer pathway is more abundant [9]. Here, we demonstrated that ANF has the same affinity as agonists to AhR as PAC. Furthermore, we reported that BNF and DMB could transactivate AhR. These results clearly demonstrate the potential

implication of these drugs in CC therapy. Synergic studies about signal transduction after AhR activation still needed to confirm this therapeutic implication.

The role of PXR in cancer is controversial too. More evidences point towards the implication of PXR activation in pro-carcinogenesis and cell growth through multiple specific pathways [10-13]. Conversely, few studies show the implication of PXR activation in anti-cancer [14]. Verma and collaborators demonstrated that PXR activation could induce apoptosis in breast cancer cells upon NO-dependent up-regulation of p53 [15]. Considerable attention is being paid to the development of PXR inhibitors. PXR antagonists could be considered as potential anti-cancer therapeutics. Here, we demonstrated that BNF could strongly antagonize with PXR. Knowing that PXR activation in oncogenesis could implicate several pathways including reactive oxygen species system activation and the modulation of pro-apoptotic gene levels [12], makes from BNF a potential potent anti-CC agent. Some studies consolidate our speculations [16], because they proved the power of BNF in lung tumor

chemoprevention in mice [17], and in suppression of induced mammary carcinogenesis [18]. This approach to block PXR activation appears to reduce number of cancer types [19,20].

Elsewhere, PAC is currently used as chemotherapeutic drug in CC in combination therapy either with cisplatin and ifosfamide or only with cisplatin [21,22]. In this study, we have demonstrated that ANF, the estrogen metabolic inhibitor, shares some homologies with PAC. Indeed, ANF transactivate with the same affinity AhR. Conversely, ANF does not transactivate PXR in PXR 4.3 cell lines with the same affinity. The above mechanisms of AhR or PXR activation represent opportunities for therapeutic intervention. Indeed, the use of PAC in CC therapy could be replaced (in patients with enhanced adverse events of paclitaxel including bone marrow suppression, cardiovascular effects, hypersensitivity and neuropathy [23]) or sustained by other efficient drugs like ANF because they transactivate identically AhR receptors. In addition, drug doses could be modulated to insure lower toxicities with higher efficiency of

cancer treatment. Recent studies support our speculation for ANF use in cancer therapy [24].

Based on the homology of PAC properties here studied, PAC could be replaced by RIF, or either could be associated to CLO or RU-486, especially because of their showed anti-cancer properties. Indeed, CLO that is clinically used as anti-fungal azole derivative has been associated with promising anti-cancer effect [25-28]. Identically, the anti-progestin, RU-486 has demonstrated powerful properties as anti-cancer drug [29-32]. Hopefully, this approach might give benefit in the future especially after further cell signaling studies.

Because PXR transactivation has been implicated in adverse drug-drug interactions [12, 33], we propose ANF as new potential therapeutic drug for CC. Indeed, this agent could efficiently activate AhR and efficiently antagonizes with PXR. This agent might be useful for postoperative chemotherapy and could reduce powerfully postoperative complications. Certainly, having demonstrated the behavior of some potential anti-cancer drugs in HeLa

transfected cell line, as agonists or antagonists of AhR or PXR, does not confirm their use in CC therapy. However, our study has initiated and guided therapeutic strategies to shed light on some particular drugs. The particular homology of AhR or PXR receptors activation or antagonism by these drugs, here demonstrated, could be the first step for the incoming studies.

AhR and PXR represent potential target tools for cancer therapy. This study may help in the design and the development of potential AhR agonists and PXR antagonists candidates offering improved avenues in CC therapy. In a continuation of this study, we sought to identify transduction signals after AhR or PXR transactivation to explain the importance of drugs here studied.

5. PERSPECTIVES

Although, AhR and PXR have been widely studied, few studies have evaluated their implication in cancer therapy. It is important to take into account the possibility of implication of such receptors as first elements in anti-cancer signaling pathways. Further studies should

concentrate on the implication of AhR and PXR transactivation or antagonism during CC therapy.

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Mohamed Soufiene Mnif

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

AhR = aryl hydrocarbon receptors

ANF = alpha-naphthoflavone

BNF = beta-naphthoflavone

CC = cervical cancer

CLO = clotrimazole

DMB = dimethoxybenzoquinone

PAC = paclitaxel

PXR = pregnane X receptors

RIF = rifampicin

REFERENCES

- [1] Garcia F, Newton J, Baldwin S. Cervical and endometrial cancer prevention, In *Fundamentals of Cancer Prevention* (Alberts, D. S., and Hess, L. M., Eds.), Berlin Heidelberg. Germany: Springer 2005; 315-347.
- [2] Jhingran A, Levenback C. Malignant diseases of the cervix. Microinvasive and invasive carcinoma: diagnosis and management, In *Katz: Comprehensive Gynecology* (Katz, V. L., Lentz, G. M., and Lobo, R. A., Eds.). Mosby: Elsevier 2007; 759-780.
- [3] Jhingran A, Russell A, Seiden M, Duska L, Goodman A, Lee S, Digumarthy S, Fuller A. Cancers of the cervix, vulva, and vagina, In *Abeloff: Abeloff's Clinical Oncology* (Abeloff, M. D., Armitage, J. O., Niederhuber, J. E., Kastan, M. B., and McKenna, W. G., Eds.) 4 ed., Churchill Livingstone: Elsevier 2008; 1745-1791.
- [4] Balaguer P, Boussioux AM, Demirpence E, Nicolas JC. Reporter cell lines are useful tools for monitoring biological activity of nuclear receptor ligands. *Luminescence* 2001; 16: 153-8.
- [5] Pillon A, Boussioux AM, Escande A, Ait-Aissa S, Gomez E, Fenet H, Ruff M, Moras D, Vignon F, Duchesne MJ, Casellas C, Nicolas JC, Balaguer P. Binding of estrogenic compounds to recombinant estrogen receptor-alpha: application to environmental analysis. *Environ Health Perspect* 2005; 113: 278-84.
- [6] Lemaire G, Mnif W, Pascussi JM, Pillon A, Rabenoelina F, Fenet H, Gomez E, Casellas C, Nicolas JC, Cavailles V, Duchesne MJ, Balaguer P. Identification of new human pregnane X receptor ligands among pesticides using a stable reporter cell system. *Toxicol Sci* 2006; 91: 501-9.
- [7] Ma C, Marlowe JL, Puga A. The aryl hydrocarbon receptor at the crossroads of multiple signaling pathways, In *Molecular, Clinical and Environmental Toxicology* (Luch, A., Ed.), Birkhäuser Verlag. Switzerland: 2009; 231-257.

- [8] Romagnolo DF, Degner SC, Selmin O. Aryl hydrocarbon receptor-mediated carcinogenesis and modulation by dietary xenobiotic and natural ligands, In *Bioactive Compounds and Cancer* (Milner, J. A., and Romagnolo, D. F., Eds.), Humana Press: 2010; 761-782.
- [9] Goergens A, Frericks M, Esser C. The arylhydrocarbon receptor is only marginally involved in the antileukemic effects of its ligand curcumin. *Anticancer Res* 2009; 29: 4657-64.
- [10] Masuyama H, Suwaki N, Tateishi Y, Nakatsukasa H, Segawa T, Hiramatsu Y. The pregnane X receptor regulates gene expression in a ligand- and promoter-selective fashion. *Mol Endocrinol* 2005; 19: 1170-80.
- [11] Masuyama H, Nakatsukasa H, Takamoto N, Hiramatsu Y. Down-regulation of pregnane X receptor contributes to cell growth inhibition and apoptosis by anticancer agents in endometrial cancer cells. *Mol Pharmacol* 2007; 72: 1045-53.
- [12] Biswas A, Mani S, Redinbo MR, Krasowski MD, Li H, Ekins S. Elucidating the 'Jekyll and Hyde' nature of PXR: The case for discovering antagonists or allosteric antagonists. *Pharm Res* 2009; 26: 1807-15.
- [13] Jiang H, Chen K, He J, Pan F, Li J, Chen J, Chen W, Liang H. Association of pregnane X receptor with multidrug resistance-related protein 3 and its role in human colon cancer chemoresistance. *J Gastrointest Surg* 2009; 13: 1831-8.
- [14] Zhang B, Cheng Q, Ou Z, Lee JH, Xu M, Kochhar U, Ren S, Huang M, Pflug BR, Xie W. Pregnane X receptor as a therapeutic target to inhibit androgen activity. *Endocrinology* 2010; 151: 5721-29.
- [15] Verma S, Tabb MM, Blumberg B. Activation of the steroid and xenobiotic receptor, SXR, induces apoptosis in breast cancer cells. *BMC Cancer* 2009; 9: 3.
- [16] Nwankwo JO. Significant transcriptional down-regulation of the human MDR1 gene by beta-naphthoflavone: a proposed hypothesis linking potent CYP gene induction to MDR1 inhibition. *Med Hypotheses* 2007; 68: 661-9.
- [17] Stoner GD, Adam-Rodwell G, Morse MA. Lung tumors in strain A

- mice: application for studies in cancer chemoprevention. *J Cell Biochem* 1993; Suppl 17F: 95-103.
- [18] Malejka-Giganti D, Bennett KK, Culp SJ, Beland FA, Shinozuka H and Bliss RL. Suppression of 7,12-dimethylbenz[a]anthracene-induced mammary carcinogenesis by pre-initiation treatment of rats with beta-naphthoflavone coincides with decreased levels of the carcinogen-derived DNA adducts in the mammary gland. *Cancer Detect Prev* 2005; 29: 338-47.
- [19] Huang H, Wang H, Sinz M, Zoeckler M, Staudinger J, Redinbo MR, Teotico DG, Locker J, Kalpana GV, Mani S. Inhibition of drug metabolism by blocking the activation of nuclear receptors by ketoconazole. *Oncogene* 2007; 26: 258-68.
- [20] Venkatesh M, Wang H, Cayer J, Leroux M, Salvail D, Das B, Wrobel JE, Mani S: In vivo and in vitro characterization of a first-in-class novel azole analog that targets pregnane X receptor activation. *Mol Pharmacol* 2011; 80: 124-35.
- [21] Hosaka M, Watari H, Kato T, Odagiri T, Konno Y, Endo D, Mitamura T, Kikawa S, Suzuki Y, Sakuragi N. Clinical efficacy of paclitaxel/cisplatin as an adjuvant chemotherapy for patients with cervical cancer who underwent radical hysterectomy and systematic lymphadenectomy. *J Surg Oncol* 2012; 105(6): 612-6.
- [22] Qiao WJ, Cheng HY, Li CQ, Jin H, Yang SS, Li X, Zhang YY. Identification of pathways involved in paclitaxel activity in cervical cancer. *Asian Pac J Cancer Prev* 2011; 12: 99-102.
- [23] Huang TC, Campbell TC. Comparison of weekly versus every 3 weeks paclitaxel in the treatment of advanced solid tumors: A meta-analysis. *Cancer Treat Rev*, 2011.
- [24] Mense SM, Singh B, Remotti F, Liu X, Bhat HK. Vitamin C and alpha-naphthoflavone prevent estrogen-induced mammary tumors and decrease oxidative stress in female ACI rats. *Carcinogenesis* 2009; 30: 1202-8.
- [25] Meira DD, Marinho-Carvalho MM, Teixeira CA, Veiga VF, Da Poian AT, Holandino C, de Freitas MS, Sola-Penna M. Clotrimazole decreases human breast cancer cells

- viability through alterations in cytoskeleton-associated glycolytic enzymes. *Mol Genet Metab* 2005; 84: 354-362.
- [26] Rodriguez-Enriquez S, Marin-Hernandez A, Gallardo-Perez JC, Carreno-Fuentes L, Moreno-Sanchez R. Targeting of cancer energy metabolism. *Mol Nutr Food Res* 2009; 53: 29-48.
- [27] Liu H, Li Y, Raisch KP. Clotrimazole induces a late G1 cell cycle arrest and sensitizes glioblastoma cells to radiation in vitro. *Anticancer Drugs* 2010; 21: 841-9.
- [28] Ahmad M. Study on cytochrome P-450 dependent retinoic acid metabolism and its Inhibitors as potential agents for cancer therapy. *Sci Pharm* 2011; 79: 921-35.
- [29] Ramondetta LM, Johnson AJ, Sun CC, Atkinson N, Smith JA, Jung MS, Broaddus R, Iyer RB, Burke T. Phase 2 trial of mifepristone (RU-486) in advanced or recurrent endometrioid adenocarcinoma or low-grade endometrial stromal sarcoma. *Cancer* 2009; 115: 1867-74.
- [30] Ørbo A, Moe BT, Grønaas H, Paulssen RH. Early effects of high concentrations of progesterone and mifepristone A gene expression study of endometrial cancer cells (Ishikawa). *J Steroid Biochem Mol Biol* 2009; 113: 139-49.
- [31] Fjelldal R, Moe BT, Ørbo A, Sager G. MCF-7 cell apoptosis and cell cycle arrest: non-genomic effects of progesterone and mifepristone (RU-486). *Anticancer Res* 2010; 30: 4835-40.
- [32] Benad P, Rauner M, Rachner TD, Hofbauer LC. The anti-progestin RU-486 inhibits viability of MCF-7 breast cancer cells by suppressing WNT1. *Cancer Lett* 2011; 312: 101-8.
- [33] Chen J, Raymond K. Roles of rifampicin in drug-drug interactions: underlying molecular mechanisms involving the nuclear pregnane X receptor. *Ann Clin Microbiol Antimicrob* 2006; 5: 3.

Table 1. Effect of anti-cancer drugs on AHR- or PXR-positive HeLa cell lines

Expressed receptor Cell line	AHR		PXR	
	AHRE 1.15		PXR 4.3	
	Agonist	Antagonist	Agonist	Antagonist
Anti-Cancer drugs				
<i>Benzoflavones</i>				
<i>alpha-naphthoflavone</i>	+++	NT	++	-
<i>beta-naphthoflavone</i>	++	+++	-	+++
<i>Clotrimazole</i>	NT	NT	++	NT
<i>Dimethoxybenzoquinone</i>	+	NT	-	-
<i>Paclitaxel</i>	+++	NT	+++	NT
<i>Rifampicin</i>	NT	NT	+++	NT
<i>RU-486</i>	NT	NT	++	NT

Abbreviations: -, no effect; +, low effect at $10^{-5}M$; ++, 50% of effect at $>10^{-6}M$; +++, 100% of effect at $<10^{-5}M$; NT, Not tested.

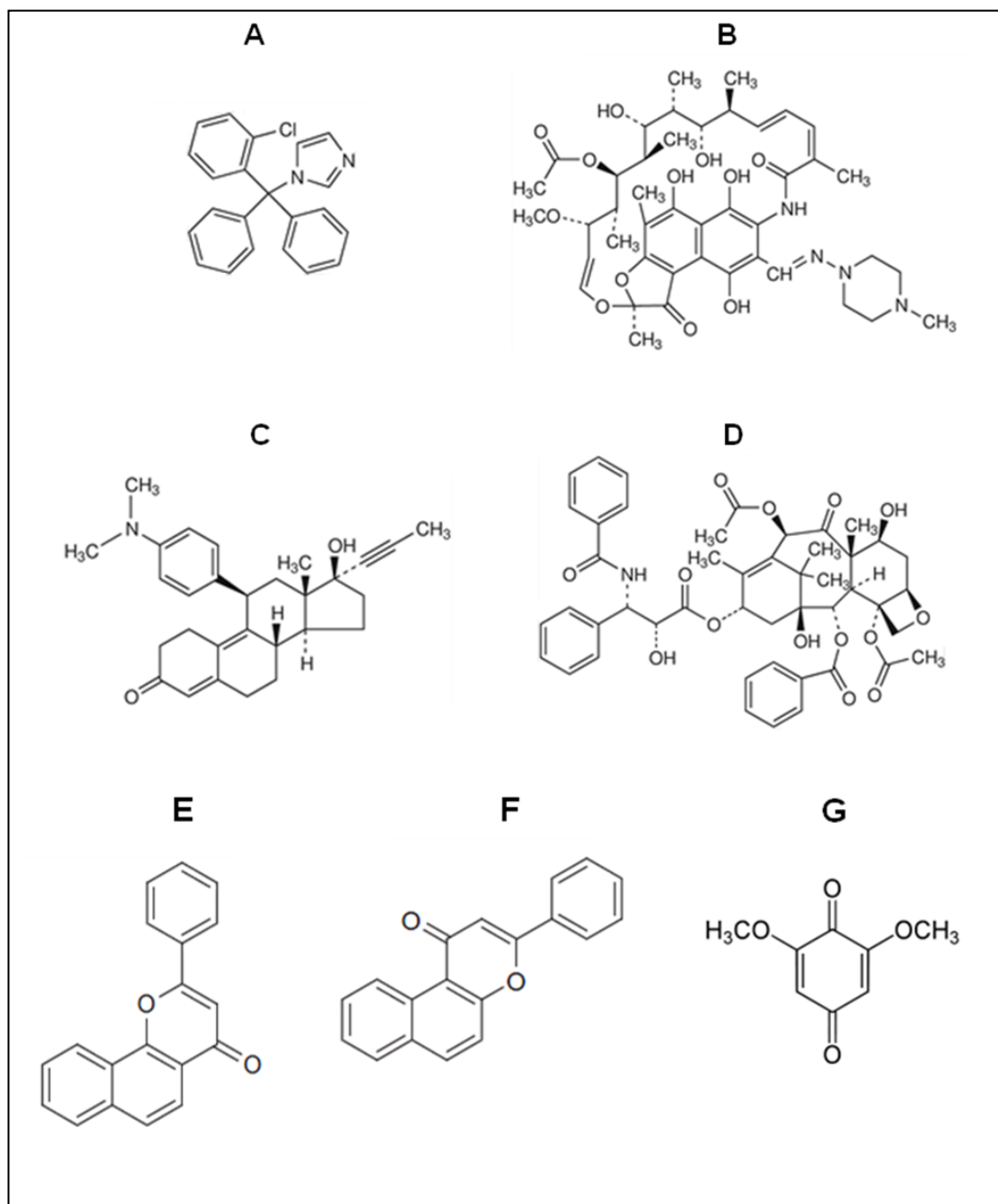


Fig. (1). Chemical structure of used anti-cancer drugs. Clotrimazole (A), Rifampicin (B), RU-486 (C), Paclitaxel (D), alpha-naphthoflavone (E), beta-naphthoflavone (F), and Dimethoxybenzoquinone (G).

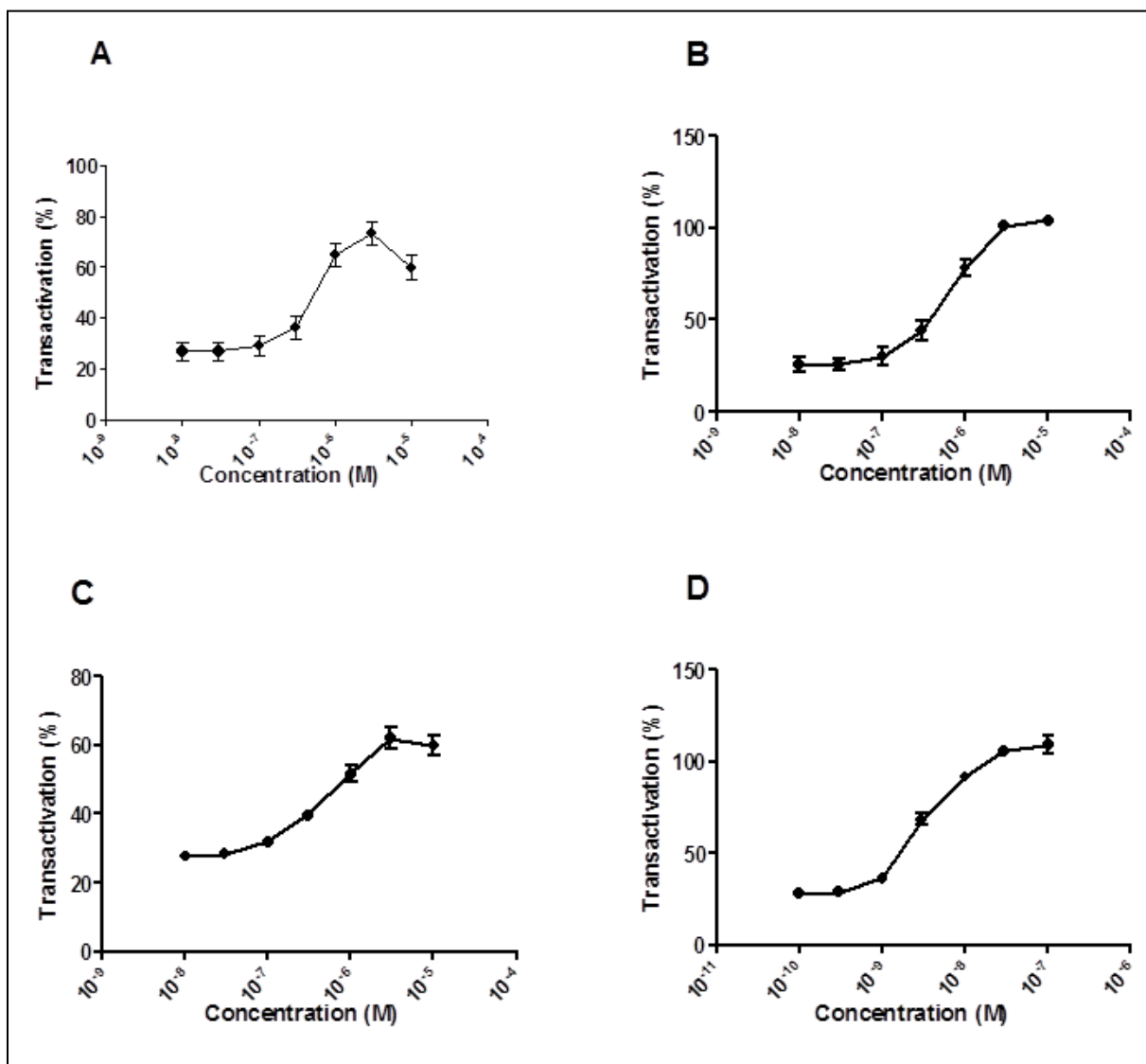


Fig. (2). Dose-response curves of the AhRE 1.15 cells and PXR 4.3 cells to human anti-cancer drugs. PXR transactivation in AhRE 1.15 cells by Clotrimazole (A), Rifampicin (B), and RU-486 (C). AhR transactivation in PXR 4.3 cells by Paclitaxel (D). After the cells incubation during 24h, percentage of luciferase activity is measured in each well. Triplicate wells were performed for each drug. Values represent means \pm S.D. of the activity of three independent experiments.